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Research Article

Simplified pipelines for genetic engineering of mammalian embryos by CRISPR-Cas9 electroporation†

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Abstract

Gene editing technologies, such as CRISPR-Cas9, have important applications in mammalian embryos for generating novel animal models in biomedical research and lines of livestock with enhanced production traits. However, the lack of methods for efficient introduction of gene editing reagents into zygotes of various species and the need for surgical embryo transfer in mice have been technical barriers of widespread use. Here, we described methodologies that overcome these limitations for embryos of mice, cattle, and pigs. Using mutation of the *Nanos2* gene as a readout, we refined electroporation parameters with preassembled sgRNA-Cas9 RNPs for zygotes of all three species without the need for zona pellucida dissolution that led to high-efficiency INDEL edits. In addition, we optimized culture conditions to support maturation from zygote to the multicellular stage for all three species that generates embryos ready for transfer to produce gene-edited animals. Moreover, for mice, we devised a nonsurgical embryo transfer method that yields offspring at an efficiency comparable to conventional surgical approaches. Collectively, outcomes of these studies provide simplified pipelines for CRISPR-Cas9-based gene editing that are applicable in a variety of mammalian species.

Summary Sentence

Efficient gene editing in mouse, pig, and cattle embryos by delivery of CRISPR-Cas9 reagents with electroporation.

Key words: embryo, CRISPR-Cas9, Nanos2, mouse, pig, cattle.

Introduction

Adaptation of the CRISPR-Cas9 system as a tool to edit genes in mammalian cells has dramatically improved the efficiency and sophistication for genetic engineering of animals [1–3]. In particular is the capacity to generate novel models for biomedical research as well as engineer livestock with enhanced production traits [4, 5]. Despite the relative simplicity of techniques for designing and producing CRISPR-Cas9 reagents for editing, the delivery of these into mammalian zygote stage embryos can be challenging. The most common approach for introducing foreign nucleic acids into mammalian embryos is microinjection which requires a level of technical expertise that is often times difficult to master, especially with livestock species [6, 7].

To overcome the difficulties of microinjection, several recent studies have devised an electroporation-based protocol for introduction of CRISPR-Cas9 reagents into mouse embryos [8–15], most notably are the methodologies termed CRISPR-EZ and GEEP that

have been devised for mouse and pig embryos, respectively [10, 11, 15]. Although conducted initially with Cas9 mRNA, a switch to use of Cas9 protein in complex with single-guide RNAs (sgRNAs) as RNPs has proven to produce significantly less mosaicism and enhance editing efficiency [9, 12, 13, 15, 16]. While a variety of mutations are effectively induced in mouse and pig embryos using these protocols, the applicability to other mammalian species such as cattle has not fully been assessed. Also, several of these protocols utilize partial dissolution of the zona pellucida to facilitate entry of sgRNA/Cas9 RNPs into the zygote [10, 11, 16], which can have deleterious effects on embryo survival and quality [17, 18]. However, recent studies with mouse and pig embryos indicate that zona disruption is indispensable for sgRNA-Cas9 RNP electroporation to achieve effective gene editing [15, 18], but the efficiency of generating live offspring with desired edits and applicability to other mammalian species remains undefined. In addition, for mice, the generation of animals from CRISPR-Cas9-treated embryos has required surgical embryo transfer [3, 10, 11]. While this approach is effective, there are inherit challenges such as animal welfare concerns and costs/risks associated with surgery.

Here, we targeted the evolutionarily conserved germ cell-specific gene *Nanos2* to devise a pipeline using electroporation of mouse zygotes with preassembled sgRNA/Cas9 RNPs followed by embryo culture to the blastocyst stage and then nonsurgical embryo transfer (NSET) to efficiently generate mutant animals. Importantly, dissolution of the zona pellucida is not needed using our methodology. In addition, we extended the electroporation approach to editing the *NANOS2* gene in bovine and porcine zygotes for efficient generation of mutant embryos that are ready for transfer. Moreover, we optimized a genotyping protocol for determining the editing efficiency in embryos at the 2 cell (2C) for early diagnostic screening. Altogether, these methods are highly effective at carrying out CRISPR-Cas9-based gene editing in mouse and livestock embryos, and the NSET approach is a technical advance that provides an effective alternative to surgical transfer.

Materials and methods

Animals and chemicals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Washington State University. Mouse strains used in this study were inbred C57BL6/J and 129S1/svlmJ and hybrids produced as an F1 cross. All chemicals were purchased from Sigma-Aldrich Company (St. Louis, MO, USA) unless noted otherwise.

CRISPR-Cas9 reagent generation

Dual sgRNAs were designed to delete a large portion of the coding sequence (CDS) for the murine, porcine, or bovine *NANOS2* gene. Briefly, candidate sgRNAs were designed and off-target predictor scores ranked using the online software programs CRISPOR (http://crispor.tefor.net/) and CRISPR Design (http://crispr.mit.edu/). For each species, sense and antisense sgRNAs were designed to introduce a staggered double-stranded break that would result in deletion of a large portion of the CDS. To achieve this, sgRNAs were designed to target ~20 nucleotides upstream of the PAM sequences in the sense or antisense strand and adjacent to the start or stop codon. All selected sgRNAs had an off-target predictor score of >80. The chosen sgRNAs targeting murine *Nanos2* were generated through a multistep process. First, PCR reactions (using DreamTaq, Thermo Fisher Scientific, Waltham, MA, USA) were used to create two overlapping oligonucleotides: (1) CRISPRF (Supplementary Figure S1) that incorporates sequence specific for the desired target site, the N18–20 guide sequence, and T7 promoter sequence; and (2) a common oligonucleotide (Supplementary Figure S1) that contains the sgRNA stem loop structure for docking of Cas9. The resultant dsDNA was gel purified (Qiaquick Gel Purification kit; Qiagen, Hilden, Germany) and used as a template for synthesis of sgRNA with the MEGAshortscript™ Kit (Thermo Fisher Scientific). Mature sgRNAs were cleaned up (Turbo Dnase, Thermo Fisher Scientific) and purified using the MEGAclear™ Kit (Thermo Fisher Scientific) and eluted at ~2000 ng/μl in TE buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 7.4). Single-guide RNAs targeting porcine and bovine *NANOS2* were designed using the online software tool and procured from a commercial source (Synthego Inc.). For all sgRNAs, aliquots were stored at –80°C for up to 6 months.

For generation of RNP complexes, sgRNAs (100 ng/μl each) and Cas9 protein (200 ng/μl) were diluted in TE buffer at 1:1 mass ratio. Opti-MEM Reduced Serum media with no phenol red (Gibco/Thermo Fisher Scientific) was then added to provide a final reaction volume of 20 μl (containing 4000 ng of sgRNA and 4000 ng of Cas9 protein), and the mixture was incubated at room temperature for 10 min before being used as the electroporation solution. For mouse zygotes, PNABio Cas9 protein (Newbury Park, CA, USA) was used. For porcine and bovine zygotes, TrueCut™ Cas9 Protein v2 (Thermo Fisher Scientific) was used.

Generation of murine zygotes and embryo culture

Female mice (>6 weeks of age) were superovulated by treating with intraperitoneal injections of PMSG (7.5 IU) followed by hCG (7.5 IU) 48 h later. Females were then paired overnight with male mice (~9 week of age) and checked for copulatory plugs the next morning. If present, cumulus–oocytes complex (COCs) at 0.5 days post conception (dpc) were recovered in DMEM/F-12 HEPES medium (Thermo Fisher Scientific) with 10% FBS from dissected ampulla. Cumulus cells were removed by manual pipetting of the zygotes in DPBS (Thermo Fisher Scientific) containing 0.1% hyaluronidase. Denudated zygotes were then washed twice in DPBS and cultured in equilibrated EmbryoMax KSOM medium at 37°C in atmosphere with 5% of CO_2 in air for 30 min prior to electroporation.

Generation of porcine zygotes and embryo culture

Porcine COCs were purchased from DeSoto Biosciences Inc. (TN, USA) and shipped overnight in maturation media at 38.5°C. Upon arrival, COCs were washed in DPBS and placed in equilibrated BO-IVM medium (IVF Biosciences, Falmouth, UK) at 38.5°C in an atmosphere of 5% CO_2 in air. Next, ~50 COCs were placed in 450 μl of equilibrated BO-IVM medium under embryo-safe mineral oil for 40–44 h post onset of maturation. The COCs were then placed in microcentrifuge tubes containing TL-HEPES (MOFA Global, Verona, WI, USA) with 0.1% hyaluronidase vortexed for 2 min. Denudated-MII oocytes were manually selected, washed, and placed in equilibrated IVF medium (BO-IVF medium supplemented with 1 μM of Caffeine) under embryo-safe mineral oil. In preparation for in vitro fertilization (IVF), refrigerated boar semen was washed three times by centrifugation (1000 × g for 4 min) in TL-HEPES (MOFA Global) and the final pellet was suspended in 50 μl equilibrated IVF medium (standard concentration of 5 × 10^5 sperm/ml). The sperm suspension was then combined with 25–30 MII oocytes and also suspended in 50 μl equilibrated IVF medium.

The IVF tubes were incubated at 38.5°C in atmosphere with 5% of CO_2 in air for 5 h. Presumptive zygotes were then washed three times by centrifugation with TL-HEPES (MOFA Global), and ~50 were cultured in 450 μl of equilibrated BO-IVC medium (IVF Biosciences) under embryo-safe mineral oil at 38.5°C in an atmosphere of 5% CO_2 and 5% O_2. Zygotes were cultured for a time period of 18–22 h post fertilization prior to being electroporated.

Generation of bovine zygotes and embryo culture

Bovine in vitro matured COCs were purchased from DeSoto Biosciences Inc. (TN, USA). Upon arrival, around 50 COCs were washed and placed in 400 μl of equilibrated BO-IVF medium (IVF Biosciences) under embryo-safe mineral oil, in accordance with the IVF Biosciences protocol. Meanwhile, a $\frac{1}{4}$ cc straw of cryopreserved bovine semen was thawed at 37°C for 30 s and viable sperm were subsequently isolated using a Bovipure gradient (Spectrum Technology, Healdsburg, CA, USA). Briefly, thawed sperm were overlaid on a 1 ml column of 80% Bovipure and centrifuged at 500 × g for 15 min [19]. The resulting sperm pellet was washed by centrifugation (300 × g for 5 min) in TL-HEPES, and the pellet was suspended in equilibrated BO-IVF. At 20–24 h post onset of maturation, 50 μl of sperm suspension (standard concentration of 1×10^6 cells/ml) was added to 450 μl of IVF medium containing 50 matured oocytes and incubated at 38.5°C in atmosphere of 5% CO_2 in air for ~18 h (according to the protocol from the laboratory of Dr P.J. Hansen, University of Florida, http://animal.ifas.ufl.edu/hansen/ivf_protocol.shtml). Presumptive zygotes were denuded by vortexing in DPBS containing 0.1% hyaluronidase before being subjected to electroporation.

Electroporation of murine zygotes

Approximately 100–150 zygotes were washed 3X in Opti-MEM media and then suspended in a minimum volume and placed into a 1 mm electroporation cuvette containing 20 μl of electroporation solution (sgRNA-Cas9 RNP complexes in Opti-MEM). Zygotes were electroporated using a BTX T820 Square Wave Electroporation system (BTX, Harvard Apparatus, Alameda, CA, USA) with standard conditions of 30V and 3 ms/pulse X 7 (CD1, F1, and C57BL6/J) or 3 (129 svlmJ) pulses. After electroporation, the cuvette chamber was washed with 1 ml of equilibrated DMEM/F-12 HEPES medium to recover all zygotes. Embryos were then washed 3X and cultured in equilibrated EmbryoMax KSOM at 37°C in an atmosphere of 5% of CO_2 in air for 3.5 days. The 2C embryo and blastocyst rates were calculated based on the total number of electroporated zygotes at 1.5 and 3.5 days post fertilization, respectively.

Electroporation of porcine and bovine zygotes

At 18–22 h post IVF, zygotes (approximately 50–80) were washed with Opti-MEM solution and transferred to a 1 mm electroporation cuvette containing 20 μl of electroporation solution (sgRNA-Cas9 RNP complexes in Opti-MEM). Electroporation was carried out using a BTX T820 Square Wave Electroporation system. The optimal conditions were 2 pulses of 20V/3 ms pulse length and 3 pulses of 30V/3 ms pulse length for bovine and porcine zygotes, respectively. Following electroporation, embryos were recovered from the cuvette by washing with 1 ml TL-HEPES. Next, embryos were washed in TL-HEPES, then equilibrated BO-IVC 3X, and ~50 were cultured to the blastocyst stage in 450 μl of equilibrated BO-IVC medium under embryo-safe mineral oil at 38.5°C in an atmosphere of 5% CO_2 and 5% of O_2. The 2C and blastocyst rates were calculated based on the total number of zygotes electroporated at 3.5 and 7–8 days post fertilization, respectively.

Nonsurgical embryo transfer in mice

Recipient mice for transfer of electroproated zygotes were CD1 females at >6 weeks of age. A pseudopregnant state was induced by pairing females overnight with vasectomized male mice (9 weeks of age), and vaginal plugs were checked the next morning. Electroporated blastocysts at 3.5 days post fertilization were transferred by a nonsurgical procedure into the uterus of pseudopregnant recipient females at 2.5 dpc. Briefly, 20–30 electroporated blastocysts were suspended in 40 μl of equilibrated EmbryoMax KSOM medium and then drawn into a premade embryo transfer device that was affixed to a single channel 2 μl pipette set at 1.8 μl. The volume was then carefully adjusted to 2 μl creating a small air bubble at the end of the device. An unanesthetized recipient female was placed on top of a wire cage lid and allowed to grasp the wire with its front feet. The midpoint of the tail was then grasped and angled upward. Next, a glass speculum was gently placed into the vagina and the embryo transfer device containing the embryos was inserted through the speculum and passed the cervix in order to access the uterus. Approximately, 20–30 embryos were then dispensed into the uterine body by depressing the pipette plunger and the device was removed without releasing the plunger. The speculum was then gently removed.

Genotyping of embryos and mice

To assess editing of the *Nanos2* gene in murine, bovine, and porcine embryos and mouse pups, genomic DNA was obtained from a single embryo (2C, 4C, 8C, or blastocyst) or tail biopsy. Embryos were suspended in 5 μl of lysis buffer (40 mM Tris, pH 8.9, 0.9% Triton X-100, 0.9% Nonidet P-40, 0.4 mg/ml proteinase K), incubated at 65°C for 15 min, and heated to 95°C for 10 min to inactivate the proteinase K. Tail biopsies (~2 mm in length) were suspended in 75 μl of lysis buffer (25 mM NaOH and 0.2 mM EDTA) and incubated at 98°C for 1 h. Reactions were neutralized by adding an equal volume of 40 mM Tris HCl (pH 5.5) buffer. Tail DNA samples were then centrifuged (1500 × g for 3 min) and supernatant was recovered. For *Nanos2* genotyping, PCR reactions (Supplementary Figure S2) were performed using 2X KAPA2G Fast HotStart Genotyping Mix with dye (Roche, Switzerland) followed by cleanup with a Qiaquick Gel Purification kit (Qiagen). If some instances, DNA was ligated into PCR2.1 vectors (TAClonig kit, Thermo Fisher Scientific), transformed into *10B E.coli* (Zymo Research, Irvine, CA, USA), and 5–10 colonies were selected for analysis by PCR. All PCR products were visualized by agarose gel electrophoresis which allowed for detecting large (>100 bp) INDEL mutations. To assess smaller mutations and confirm large mutations, PCR products were subjected to sequencing analysis using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) followed by cleanup (Mag-Bind SeqDTR, Omega Bio-Tek, Norcross, GA, USA). Mutation detection was made by aligning base calls to published wild-type *Nanos2* DNA sequence for murine, bovine, or porcine.

Phenotyping of *Nanos2* knockout mice

Testes were collected from adult male mice at 3 months of age that possessed inactivating mutations in either one or both *Nanos2* alleles and fixed in Bouin solution for 10 h. The tissue was then embedded in paraffin and 5 μm cross-sections generated for staining with hematoxylin and eosin. The morphology of cross-sections was

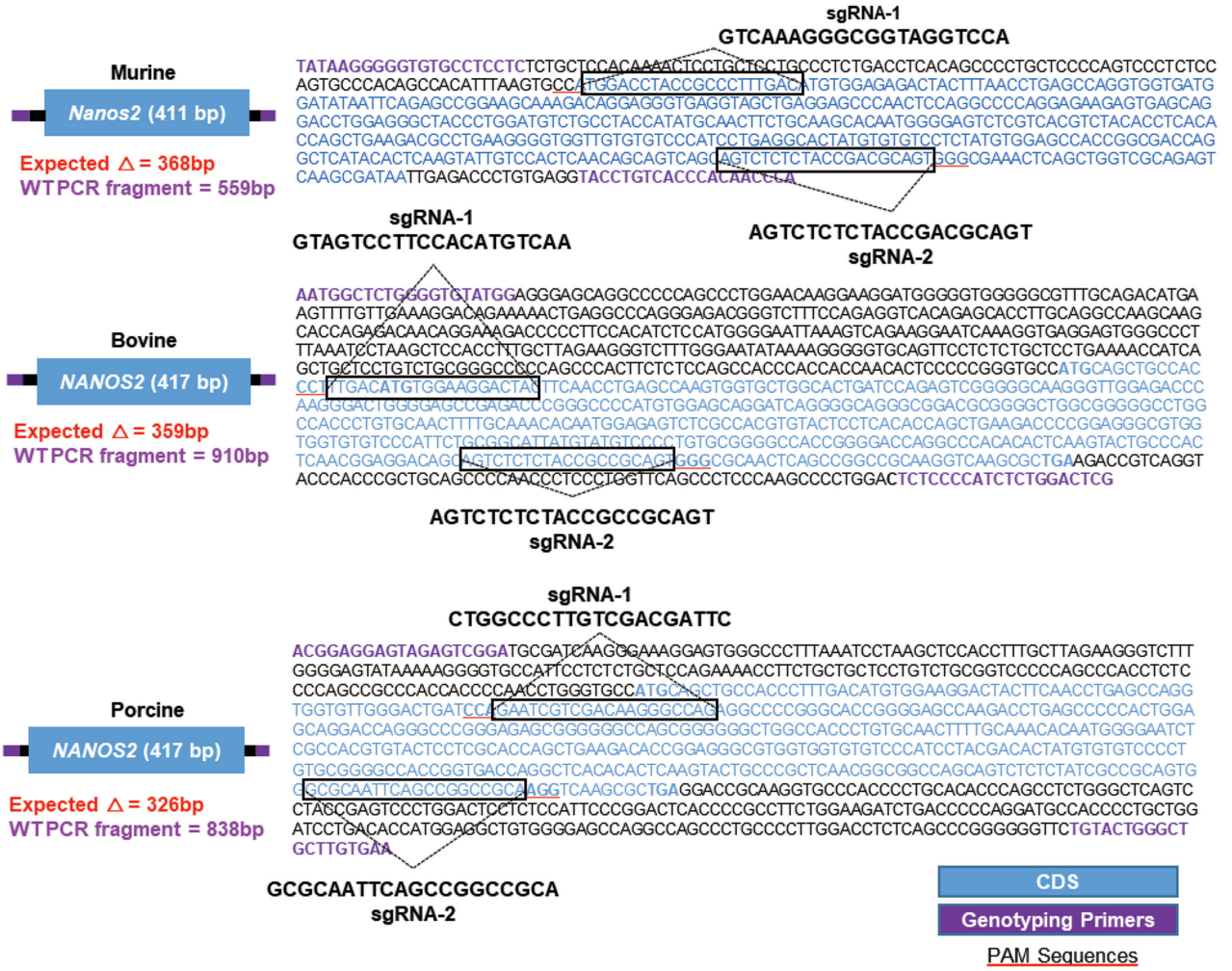


Figure 1. Design and validation of CRISPR-Cas9 reagents for editing the murine, bovine, and porcine *NANOS2* genes. Nucleotide composition of the coding sequence (depicted in blue) and 5′/3′ flanking regions of the *NANOS2* gene. Primer sites for genotyping analyses are depicted in purple, PAM sequences are underlined in red, and the dual sgRNAs are indicate by boxes.

evaluated using light microscopy and digital images captured with Olympus IX51 microscope, Olympus TH4–100 digital camera, and Cell Sense Dimensions software. The images were captured with ×400 magnification.

In vitro digestion of NANOS2 template DNA with Cas9 Nuclease

Template DNA for murine, porcine, and bovine NANOS2 were generated by PCR using the genotype parameters listed in Supplementary Figure S2 and primers described in Figure 1. The products were cleaned up with a Qiaquick Gel Purification kit (Qiagen). Next, RNP complexes were generated by incubating sgRNAs (30 nM final concentration) and Cas9 protein (30 nM final concentration; NEB # M0386, New England Biolabs, MA, USA) in BEBuffer 3.1 at 1:1 mass ratio at room temperature for 10 min. Template DNAs from each species were then added to the respective RNP complexes at the final concentration of 3 nM and incubated at 37°C for 15 min. Cleavage reactions were stopped by addition of Proteinase K for 10 min at room temperature, and DNA fragmentation patterns were visualized by agarose gel electrophoresis (2% agarose in TBE and resolved at 100 V for 1 h).

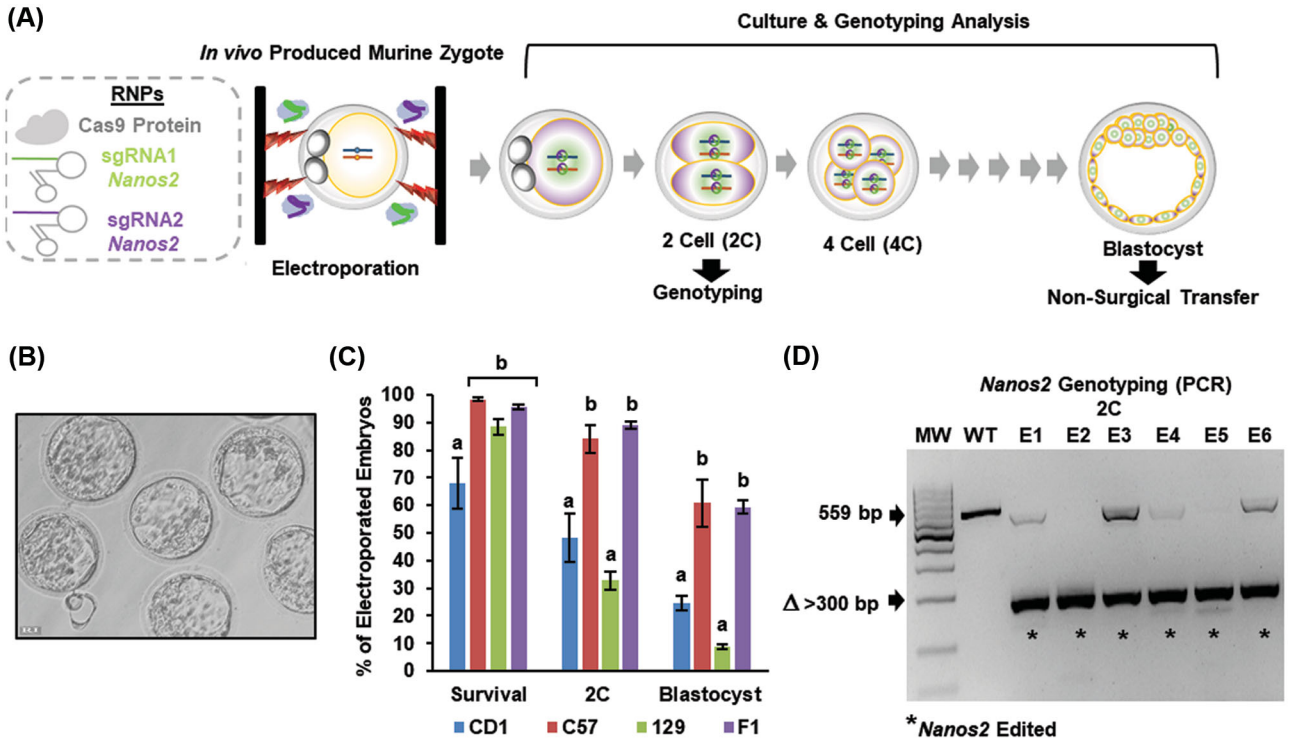
Statistical analysis

All statistical assessments were conducted with SPSS software (IBM, Armonk, NY). Outcomes of IVF (cleavage and blastocyst rates) were analyzed by one-way ANOVA test. A $P < 0.05$ was considered significantly different.

Results

Design and validation of sgRNAs targeting murine, bovine, and porcine *NANOS2* genes

To provide a simple readout for gene knockout via CRISPR-Cas9-based editing, we targeted the evolutionary conserved germ cell-specific gene *NANOS2*. The *NANOS2* gene is a single exon in all mammalian species studied with an abundance of PAM sites thereby making sgRNA design straightforward (Figure 1A). Furthermore, *NANOS2* is expressed specifically in the male germline and the only phenotype from inactivation of the gene is male sterility [20–22], thus



(E) *Nanos2* Genotyping (Sequencing): sgRNA-Cas9 RNPs

Sequence (DNA)	Embryo Edits
ATTTAAGTGCCATGGACCTACCGCCCTTTGAC...AGTCTCTCTACCGACGCAGTGGGCGAAACTCAGCTGG	WT, +/+
ATTTAAGTGCC----------TACCACCCTTTGAC...AGTCTCTCTACCGACGCAGTGGGCGAAACTCAGCTGG	E1, Δ7 bp
ATTTAAGTGC----------...----------TGG	E1, Δ390 bp
ATTTAAGTGC----------...----------TGG	E2, Δ390 bp/Δ390 bp
ATTTAAGTGCC----------TACCACCCTTTGAC...AGTCTCTCTACCGACGCTGTGGGCGAAACTCAGCTGG	E3, Δ7 bp
ATTTAAGTGC----------...----------TGG	E3, Δ390 bp
ATTTAAGTGC----------...----------TGG	E4, Δ390 bp
ATTTAAGTGC----------...----------TGG	E5, Δ390 bp/Δ390 bp
ATTTAAGTGCC----------TACCACCCTTTGAC...AGTCTCTCTACCGACGCAGTGGGCGAAACTCAGCTGG	E6, Δ7 bp
ATTTAAGTGC----------...----------TGG	E6, Δ390 bp

Figure 2. Editing efficiency of the *Nanos2* gene in murine embryos via CRISPR-Cas9 RNP electroporation. (A) Schematic of a simplified workflow for electroporating mouse zygotes followed by culture to the blastocyst stage. (B) Representative image of electroporated blastocysts at day 3.5. (C) Percentage of electroporated zygotes that advanced to the 2 cell (2C) and blastocyst stage in vitro. Data are mean ± SEM for n = 3 different batches of 30–100 embryos. Bars with different letters are significantly different at $P < 0.05$. (D) Representative image of an agarose gel from PCR-based genotyping analysis of individual 2C embryos (n = 6) that had been electroporated with *Nanos2* sgRNA-Cas9 RNPs as zygotes. MW = 100 bp molecular weight ladder and WT = wild-type control. (E) Outcomes of DNA sequencing analysis for *Nanos2* alleles from individual 2C embryos (n = 7) that were derived from zygotes electroporated with *Nanos2* sgRNA-Cas9 RNPs. For the wild-type (WT) sequence, the sgRNAs are underlined, the PAM sequences are highlighted in red, and the predicted Cas9 cut sites are highlighted in purple.

confounding effects of embryo and fetal lethality when attempting to optimize CRISPR-Cas9-based gene editing workflows are circumvented. To target inactivation of *NANOS2*, we designed pairs of sgRNAs for the murine, bovine, and porcine sequences that would delete a large portion (>300 bp) of the CDS (Figure 1A) and each was found to be highly efficient at guiding cleavage of a DNA fragment in vitro when aggregated with Cas9 protein as an RNP (Supplementary Figure S3).

Editing efficiency of the *Nanos2* gene in murine zygotes following electroporation with sgRNA-Cas9 reagents

Having devised efficacious CRISPR-Cas9 reagents for editing a *Nanos2* DNA sequence, we next aimed to determine the efficiency of gene editing within embryos (Figure 2A). For murine studies, we used superovulation and natural mating to generate zygotes. After flushing from the oviduct, zygotes (n = 100–200) were washed and

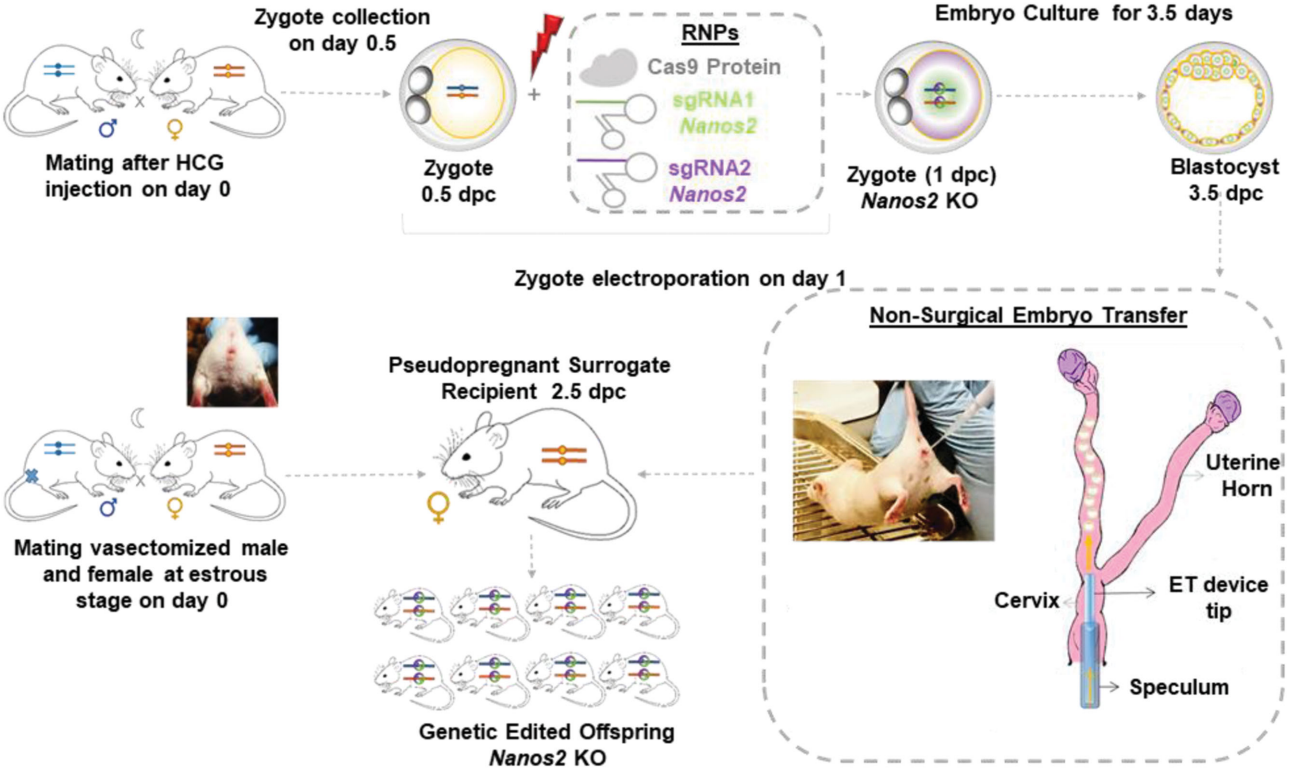


Figure 3. Schematic of zygote electroporation and nonsurgical embryo transfer (NSET) pipeline for mice.

suspended in Opti-MEM buffer and then subjected to electroporation using standard parameters of 30V, 3 ms pulse length, and 7 pulses, in conjunction with pre-assembled sgRNA-Cas9 RNPs for editing of the *Nanos2* gene. In deviation to previous studies, we did not treat zygotes for dissolution of the zona pellucida prior to electroporation. The embryos were then cultured to the blastocyst stage, and 2C embryos were used for genotyping analyses. We assessed the survival and editing efficiency of embryos derived from two different inbred strains of mice (C57BL6/J and 129S1/svlmJ), an outbred strain (CD1), and an F1 hybrid (C57BL6/J X 129S1/svlmJ). Outcomes revealed a high survival rate for C57BL6/J and F1 embryos with over 60% of those electroporated advancing to the blastocyst stage (Figure 2B and C). Although initial survival was comparable for 129S1/svlmJ embryos, the percentage that progressed to blastocyst stage (~9%) was significantly less compared to the other strains (Figure 2B and C). For CD1, the initial survival rate was reduced compared to the other strains but ~36% of the 2C embryos were able to advance to the blastocyst stage (Figure 2B and C).

In the course of assessing blastocysts, we reasoned that genotyping of earlier stage embryos might be advantageous for screening the effectiveness of CRISPR-Cas9 reagents. For example, a subset of electroporated zygotes could be genotyped at the 2C stage to assess editing efficiency before progressing the culture to morula or blastocysts for embryo transfer thus allowing for a stop-go decision point. This approach would be especially advantageous with large animals such as livestock for which having multiple stop-go decisions points would help reduce wasted costs with culturing and transferring batches of embryos that have low mutation frequency. Therefore, we explored whether PCR-based genotyping could be used for low input DNA that would be obtained from individual 2C embryos that were electroporated as zygotes. Outcomes revealed that ~90% of 2C embryos of all strains contained at least one *Nanos2* allele with an INDEL mutation of >300 bp (Figure 2D) which was confirmed by DNA sequencing analysis (Figure 2E). Collectively, these findings demonstrate high efficiency for generating INDEL mutations in mouse embryos via electroporation of sgRNA-Cas9 RNPs without zona pellucida dissolution, reveal strain differences in sensitivity to the treatment, and present a technological advance for screening batches of early-stage embryos.

Derivation of an efficacious NSET approach in mice to generate gene-edited animals

In addition to delivery of CRISPR-Cas9 reagents into zygotes and subsequent embryo culture, the efficient generation of gene-edited animals requires the transfer of embryos to surrogate females for establishing pregnancies. The conventional approach to achieving this in mice has been a surgical embryo transfer that can be costly when a large number of surgeries are performed, requires a gained level of technical skill by the investigator, is time consuming, and has animal welfare connotations. To address these nuances, we devised an efficient NSET alternative for mice (Figure 3). First, we modified a tube that would easily pass through the cervix and into the uterus of an adult mouse. This tube can easily be placed on the end of a standard pipettor for dispensing blastocyst-stage embryos that are suspended in a small drop of media into the uterus of a pseudo pregnant (day 2.5) mouse. Importantly, no anesthesia is required for the recipient female and the procedure takes less than 1 min to perform, on average.

To test the effectiveness of the NSET approach for generation of mutant mice, we transferred embryos that had been electroporated with *Nanos2* sgRNA-Cas9 RNPs as zygotes and cultured to the

blastocyst stage into CD1-recipient females. For control embryos that were not electroporated, the pregnancy rate from NSET was found to be 100% (n = 3 different ET sessions with 90 embryos and 3 recipients) and the average litter size from 30 embryos transferred per female was 10 (Figure 4A). For the *Nanos2* sgRNA-Cas9 RNP electroporated embryos, the pregnancy rate from NSET was also 100% (n = 2 different ET sessions with 54 embryos and 2 recipients) and the average litters size from 20–30 embryos transferred was ~6, which was similar to the rate we achieved by surgical ET (Figure 4A). Genotyping analysis of the individual pups born (n = 20) indicated that 70% had mutations in the *Nanos2* gene (Figure 4B), with both alleles edited in 65% and one allele edited in 5% (Figure 4C) which was confirmed by sequencing analysis (Figure 4D). Importantly, 100% of mice born from electroporated embryos transferred with the NSET approach, including those with presumptive monoallelic or biallelic inactivating edits were viable and survived to adulthood (Figure 4E). Moreover, all males determined to possess biallelic edits (i.e. knockouts, n = 9) possessed regressed testes and were germline ablated (Figure 4F and G), thus phenocopying *Nanos2* null mice that were generated previously with ES cell-based gene targeting strategies [22]. Collectively, these findings demonstrate an efficacious approach for generating mutant mice from electroporated embryos that overcomes challenges associated with surgical transfer.

Editing efficiency of the *NANOS2* gene in bovine and porcine zygotes following electroporation with sgRNA-Cas9 RNPs

Beyond the generation of mouse models for basic research, genetic engineering has major applications in producing lines of livestock for the purpose of improving production traits and disease resistance. A major hurdle with conventional approaches has been the inability to introduce recombinant DNA or other nucleic acids into the pronuclei of zygotes and lack of ES cell technology for gene targeting. Even with the advent of CRISPR-Cas9 technology, microinjection of reagents into livestock embryos is technically challenging. To address this, we explored the utility of electroporating bovine and porcine zygotes with sgRNA-Cas9 RNPs to generate INDEL mutations in the *NANOS2* gene (Figure 5A). First, we optimized a pipeline for in vitro production of zygotes that yielded fertilization rates of ~92% and ~76% for bovine (n = 3 different sessions and 200 oocytes per session) and porcine (n = 3 different sessions and 200 oocytes per session) eggs, respectively (Figure 5B and C). Second, we optimized in vitro culture conditions for maturing bovine zygotes to blastocyst-stage embryos which yielded an average efficiency of ~67% (n = 3 different sessions) (Figure 5C). Next, we explored different electroporation parameters for both bovine and porcine zygotes to optimize embryo survival rates. For these experiments, we started with a standard of 30V for 3 ms and 2 pulses and assessed the percentage of 2C and 8C or blastocyst-stage embryos that derived from bovine and porcine zygotes, respectfully. In these conditions, porcine embryo survival was not different compared to the nonelectroporated control condition (Figure 5B), whereas bovine embryo survival was significantly reduced compared to the control (Figure 5C). Thus, we examined whether porcine zygotes could withstand harsher conditions with a third pulse and again found no differences in either 2C or 8C survival rate compared to the control condition (Figure 5B). For bovine, we explored other voltages and found that 2C and blastocyst survival was not different compared to the control condition at 20V (Figure 5C). For these reasons, we chose conditions of 30V-3ms-3 pulses and 20V-3ms-2 pulses to explore *NANOS2* gene editing in bovine and porcine embryos, respectively. At present, culture conditions that support progression of porcine embryos to the blastocyst stage have not been optimized; thus, we were unable to assess in vitro survival and development beyond the 8C stage following electroporation. Moreover, we observed that reduced osmolarity of electroporation buffer was deleterious to embryo development (Figure 5B and C).

Lastly, we tested the editing efficiency of generating INDEL mutations in *NANOS2* of porcine embryos at the 2C-8C stage and bovine embryos from 4C to blastocyst stage that had been developed in vitro from zygotes with sgRNA-Cas9 RNPs. Outcomes from conventional PCR-based genotyping of individual embryos revealed that ~63% (n = 22) contained INDEL mutations of >300 bp in at least one *NANOS2* allele for bovine and porcine (Figure 5D–I), and the mutations were confirmed by DNA sequencing analysis (Supplementary Figure S4). Importantly, the sequencing analysis revealed that both *NANOS2* alleles were edited in 73 and 82% of embryos for porcine (n = 11) and bovine (n = 20), respectively (Figure 5F and I). Collectively, these results demonstrate an efficient pipeline for CRISPR-Cas9-based gene editing to alter alleles in livestock embryos that are ready for embryo transfer.

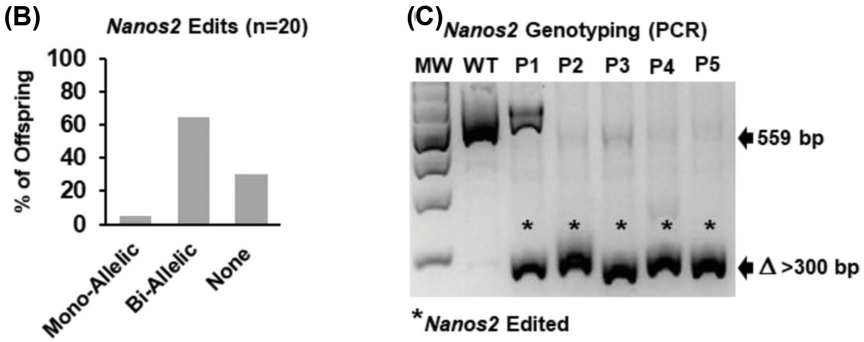
Discussion

The application of gene editing methodologies for the generation of animals is far reaching, ranging from the creation of new biomedical research models to better understand the etiology of diseases to generation of livestock with enhanced traits that are important for meat, milk, and fiber production [4, 5, 23, 24]. Regardless of the application, efficient pipelines are essential to reduce costs, decrease the timeframe for obtaining the desired genotype, and improve animal welfare. These aspects are especially important for application in large animals that have long generation intervals and extended postnatal age periods to puberty. Although previous studies have devised effective strategies for applying CRISPR-Cas9-based gene editing in mouse embryos, few have explored extension to livestock. In addition, studies focused on the generation of mouse models have employed surgical embryo transfer as a means to produce animals from gene-edited embryos. While effective, this approach can pose technical and animal welfare challenges.

In the current study, we confirm the outcomes of previous reports [10, 11, 16, 18, 25] that electroporation of mouse zygotes with CRISPR-Cas9 RNPs is highly effective at generation of INDEL mutations. Importantly, we have advanced the methodology by confirming that zona pellucida dissolution, even partial, is not needed for delivery of the sgRNA-Cas9 RNPs in mouse and pig embryos [15, 18], and extend this to bovine embryos. This outcome is a major advance to the area of animal genetic engineering because the approaches used in previous studies such as protease or acid Tyrode treatment or pre-electroporation can have deleterious effects on embryo survival and quality [10, 11, 16–18, 26]. In addition, our results provide other significant advances including an ability to genotype individual embryos at the 2C stage and mature the electroporated embryos to the blastocyst stage with high efficiency. Moreover, we demonstrate an optimized method of NSET that yields live mutant offspring at an efficiency comparable to surgical embryo transfer. Our approach provides an effective alternative to NSET methodology reported previously [27], which does not require the purchase of a commercial device and should be useful by any lab with a basic understanding of mouse handling and reproductive biology. Although

(A)

Method	Target Gene	Donor Mouse Strain	Embryos (M+B)	Litter Size	Mutant	Bi-Allelic	Mono-Allelic
Surgery	*Nanos2*	CD1	30	6	6	5	1
	Nanos2	CD1	30	3	3	3	0
NSET	*Nanos2*	129	28	6	2	2	0
	Nanos2	F1	26	5	3	3	0
	Control 1	F1	30	9	-	-	-
	Control 2	F1	30	11	-	-	-
	Control 3	F1	30	10	-	-	-



(D) *Nanos2* Genotyping (DNA Sequencing) - Pups

GTGCCATGGACCTACCGCCCTTTGA...AGTCTCTCTACCGACGCAGTGGGCGAAACTCAGCTGG	WT, +/+
GTGCCATGGA----------...----------AGTGGGCGAAACTCAGCTGG	P1,+/Δ367bp
GTGCCATGG----------...----------GAAGTGGGCGAAACTCAGCTGG	P2, Δ366bp/Δ366bp
GTGCCATGG----------...----------GCGAAACTCAGCTGG	P3, Δ373bp/Δ373bp
GTGCCATGGA----------...----------AGTGGGCGAAACTCAGCTGG	P4, Δ368bp/Δ368bp
GTGCCATGGA----------...----------AGTGGGCGAAACTCAGCTGG	P5, Δ367bp/Δ367bp

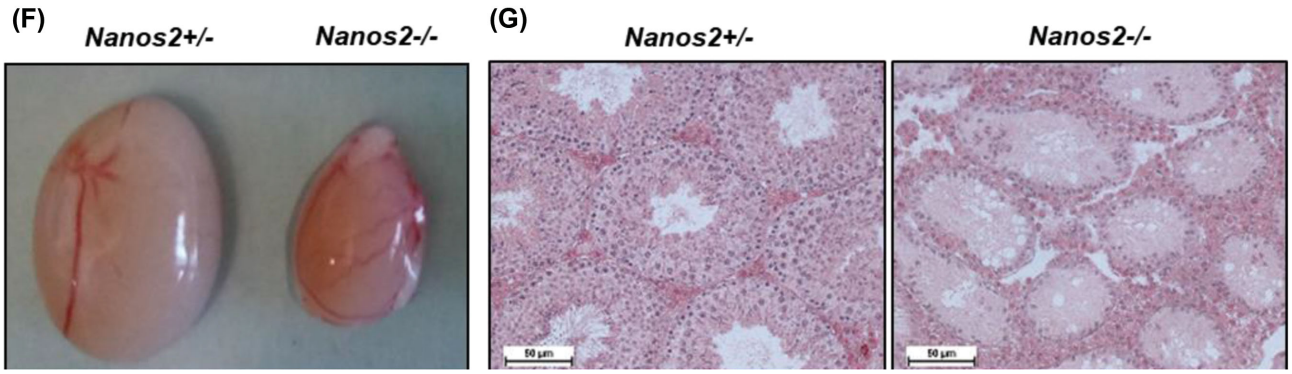


Figure 4. Outcomes of generating gene-edited mice from CRISPR-Cas9 electroporated zygotes and NSET. (A) Summary of pregnancy rates and litter size for three different NSET sessions. All recipients were CD1 females and the embryo donors were CD1, 129S1/svImJ, or an F1 hybrid of C567BL6/J X 129S1/svImJ. (B) Percentage of offspring determined to have one allele (monoallelic), both alleles (biallelic), or neither allele (none) edited by PCR-based genotyping analysis. Data are mean ± SEM for n = 4 different batches of 20–30 embryos. (C) Representative image of PCR-based genotyping analysis for *Nanos2* gene edits in individual pups born by NSET following zygote electroporation. MW = 100 bp molecular weight ladder and WT = wild-type control. (D) Outcomes of DNA sequencing analysis for pups generated by zygote electroporation and NSET (n = 6) that were genotyped by PCR as having b-allelic editing. For the wild-type (WT) sequence, the sgRNAs are underlined, the PAM sequences are highlighted in red, and the predicted Cas9 cut sites are highlighted in purple. (E) Representative image of an NSET recipient mouse with *Nanos2* gene-edited pups. (F and G) Representative images of testes (F) and cross sections of seminiferous tubules (G) from *Nanos2* +/– and *Nanos2*–/– mice generated by CRISPR-Cas9 zygote electroporation and NSET. Note that the *Nanos2*–/– testes are regressed and the germline is ablated within seminiferous tubules.

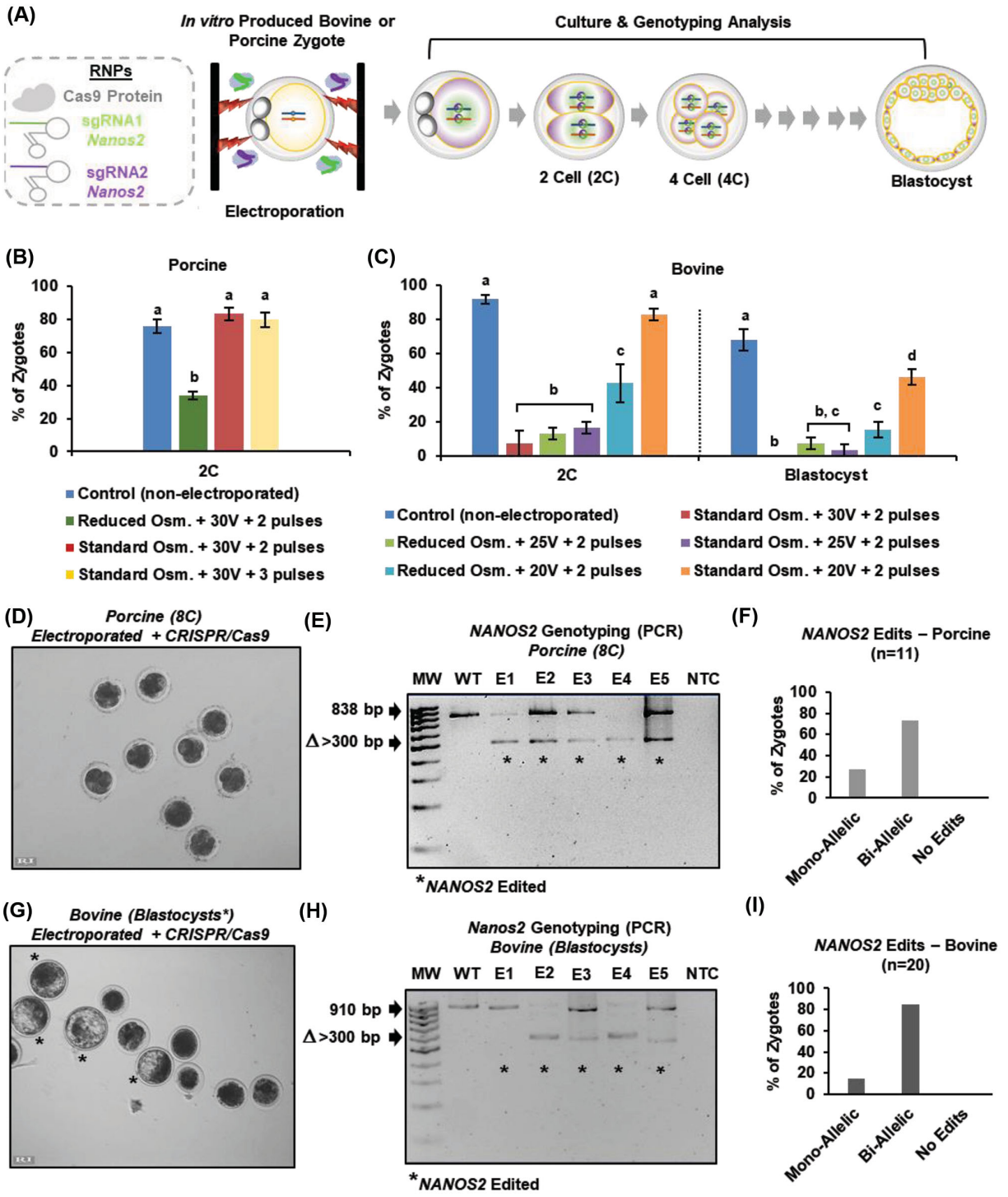


Figure 5. Editing efficiency of the *NANOS2* gene in bovine and porcine embryos via CRISPR-Cas9 electroporation. (A) Schematic of a simplified workflow for electroporation of in vitro produced bovine and porcine zygotes followed by culture to the blastocyst stage. (B and C) Percentage of porcine (B) and bovine (C) zygotes that progressed to the 2 cell (2C) and blastocyst stages in vitro following treatment with different electroporation parameters or no treatment (nonelectroporated control). Data are mean ± SEM for n = 5 (bovine) and n = 4 (porcine) different batches of 40–60 embryos each. Bars with different letters are significantly different at $P < 0.05$. (D) Representative images of 8 cell (8C) porcine embryos at 3.5 days post electroporation. (E) Representative images of PCR-based genotyping analysis for *NANOS2* edits in individual porcine 8C embryos that had been electroporated with sgRNA-Cas9 RNPs at the zygote stage. MW = 100 bp molecular weight ladder. WT = wild-type control and NTC = no template control. (F) Percentage of porcine 8C embryos with one allele (monoallelic), both alleles (biallelic), or neither allele (none) edited as determined by PCR-based genotyping analysis. Data are mean ± SEM for n = 11 embryos. (G) Representative images of bovine embryos 7.5 days post electroporation. (H) Representative images of PCR-based genotyping analysis for *NANOS2* edits in individual bovine blastocysts that had been electroporated with sgRNA-Cas9 RNPs at the zygote stage. MW = 100 bp molecular weight ladder. WT = wild-type control, and NTC = no template control. (I) Percentage of bovine blastocysts with one allele (monoallelic), both alleles (biallelic), or neither allele (none) edited as determined by PCR-based genotyping analysis. Data are mean ± SEM for n = 20 embryos.

100% of recipient mice became pregnant using our NSET approach in the current study, only a few procedures were performed (n = 5 recipients); thus, the rate may be less when applying the technique on a larger scale.

Although genetic engineering has been lauded for decades as a prime application in livestock for generating animals with enhanced production traits that will be beneficial for human consumption, a roadblock has been lack of ability to efficiently introduce reagents for modifying the genome into embryos [6, 7]. Even with the advent of gene editing technologies, introduction of reagents into livestock zygotes via microinjection has limited efficiency and requires skills that are often difficult to master. In the current study, we demonstrate high efficiency of generating INDEL mutations in bovine and porcine zygotes via electroporation. Interestingly, we found that bovine zygotes are more sensitive to electroporation conditions than porcine zygotes. The reason for this is unclear but highlights a need for tailoring conditions to each species of interest. Importantly, the mutant embryos generated via electroporation are able to be developed in vitro to the blastocyst stage, also at a high efficiency. In both cattle and pigs, ET is conducted with morula or blastocyst-stage embryos; thus, our workflow for gene editing of livestock embryos is well matched with standard advanced reproductive techniques in the field for generating engineered animals.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary Figure S1. Oligonucleotide sequences used to generate murine sgRNAs.
Supplementary Figure S2. PCR program for embryo (2C to blastocyst) genotyping.
Supplementary Figure S3. (**A-C**) In vitro assessment of the efficiency of sgRNA-Cas9 RNPs at cleaving murine (A), bovine (B), and porcine (C) *NANOS2* DNA fragments. MW = 100 bp molecular weight ladder.
Supplementary Figure S4. DNA sequencing analysis of *NANOS2* alleles in porcine 8C embryos (A) and bovine blastocyts (B) to confirm edits. For the wild-type (WT) sequences, the sgRNAs are underlined, the PAM sequences are highlighted in red, and the predicted Cas9 cut sites are highlighted in purple.

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Author contributions

DM, MIG, MC, BLB, and JMO conceived of and designed the study and interpreted the results. DM and JMO conceived the NSET approach. DM performed the NSET approach. JMO wrote the manuscript with input from all of the other authors.

Conflict of interest: The authors have declared that no conflict of interest exists

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